Chapter 3: Bioactivity-guided purification and identification of anti-HIV molecule in Black Clam crude extracts
3.1 Introduction

A large number of bioactive molecules have been isolated from marine organisms (Donia et al, 2003). The crude extracts having biological activity are used as a source for isolation of active molecule(s) using different methods of purification. The extract is subjected to several rounds of fractionation to separate other molecules from bioactive molecule. The highly pure fraction with potent biological activity is then subjected to structural analysis. One of the popular approaches is to use combination of methods of purification, such as HPLC separation and other chromatographic methods followed by determination of the structure by Mass spectroscopic analysis, Nuclear magnetic resonance (NMR), Infrared (IR) and X-ray crystallography. Majority of the biologically active natural products have been isolated using bioactivity-guided fractionation (Pezzuto et al, 1997). In bioactivity-guided fractionation, the extract of an organism or a mixture of unknown molecules is fractionated and simultaneously biological activities of purified fractions are tested to determine the active fraction in each step of purification. In this process, extract of an organism having large number of molecules is initially separated into two or major parts based on their solubility in aqueous and organic solvents or a combination of organic and aqueous solvents. Then the bioactive sample is further purified into small fractions using chromatographic methods and HPLC, etc. Purified fractions in each step of purification are subjected to biological activity testing. This procedure is also useful to detect any modifications in the nature of bioactive component due to the purification, which may lead to the loss of its bioactivity. This procedure is also useful to select and make changes in the process of purification to purify the active molecule without significant changes in its activity.

The first two phases in elucidation of the structure of molecules are identification of functional groups and carbon skeleton. The main objective of spectroscopic analysis is to identify functional groups in the molecule and molecular fragments. In this, majority of structural correlations are obtained empirically by examining the spectra of known compounds. Mass spectroscopy is useful to calculate the
accurate mass of small molecules, peptides and proteins etc. as well as to identify chemical entities in the structure of the molecule. NMR is used to give information on detailed environment of the nucleus and its relationship to its neighbors. In this, chemical shift of a proton resonance is a reflection of magnetic environment of the nucleus. IR spectrum is important to reveal details of functional groups and also interaction between functional groups in the molecule. However, structural conclusions are drawn from various methods using complementary information regarding the structure of the molecule. Use of two or more complementary methods of structural analysis provide valuable structural details necessary for definitive assessment of identity and to ensure a high degree of experimental confidence.

The preparation of crude extracts of Indian marine bivalves and their anti-HIV-1 property was described in previous chapter. In all antiviral-screening experiments, Black Clam crude extracts showed potent anti-HIV-1 activity as compared to other extracts of Indian marine bivalves. The Black Clam crude extract was selected for purification of active molecule. The bioactive molecule with anti-HIV-1 activity was isolated from Black Clam crude extract by bioactivity-guided fractionation and was identified as Zinc diprolinate using NMR and Mass spectroscopic analysis. Zinc diprolinate was synthesized and antiviral screening assays indicated that it is a potent inhibitor of HIV-1 replication.

3.2 Materials and methods

3.2.1 Cell culture

U937 and THP-1 human monocytic cell lines were obtained from NCCS cell repository. CEM-GFP-CCR5, a CCR5 receptor expressing CEM-GFP cell line (Ladha et al, 2005) as well as U937 and THP-1 cell lines were maintained in RPMI 1640 (Invitrogen, USA) with 10% fetal bovine serum (FBS) (Invitrogen, USA). The antibiotics, 100 U/ml of penicillin and 100 μg/ml of streptomycin (Invitrogen, USA) were added to avoid bacterial contamination. G418 sulphate (500 μg/ml) (Invitrogen, USA) and Zeocin (600 μg/ml) (Invitrogen, USA) were added to the medium of CEM-GFP-CCR5 cells as selection antibiotics. Human
peripheral blood mononuclear cells (hPBMCs) were isolated using Ficoll-Hypaque solution (Amersham biosciences, USA) according to the manufacturer’s protocol and were cultured in RPMI 1640 (Invitrogen, USA) with 10% fetal bovine serum (FBS) (Invitrogen, USA) and 20 U/ml of human interleukin 2 (hIL-2). The other methods of cell culture and experimental details performed were as described in section 2.2.

3.2.2 Virus isolates, HIV-1 infection and anti-HIV-1 screening assay

HIV-1\textsubscript{Indie-C1}, an infectious full-length molecular clone of HIV-1 subtype C Indian isolate was a kind gift of Dr. M. Tatsumi, Japan (Mochizuki et al, 1999). Viral stocks were generated as described in section 2.2.4. HIV-1\textsubscript{L10R/M461/I63P/V82T}, a protease inhibitor resistant viral isolate and HIV-1\textsubscript{JF4A}, a ddC drug resistant viral isolate were obtained from NIH AIDS reagent program, USA. HIV-1 infection of CEM-GFP, Jurkat, U937 and THP-1 cells was performed as described in section 2.2.6. For infection of U937 and THP-1 cells, 0.5 MOI of HIV-1 NL4-3 was used. The primary subtype C Indian isolates were obtained from virus repository of National AIDS Research Institute (NARI), India. For infection, 5 ng of primary isolate was used to infect 2.5 million hPBMCs.

3.2.3 Solvent extraction of the crude extract

The crude extract of Black Clam was separated into two major parts using solvent extraction method. In this, water and methanol were used as solvents to extract water and methanol soluble components of crude extract. The lyophilized Black Clam crude extract was dissolved in methanol with stirring using a magnetic stirrer for 3 to 4 hours. The solution was then allowed to settle at room temperature to separate methanol-insoluble residue and a clear supernatant of methanol soluble fraction. Methanol soluble fraction and insoluble residue were separated using a Whatman filter paper. Then methanol-insoluble residue was air-dried and was dissolved in double distilled water to prepare 100 mg/ml of
aqueous fraction. The methanol soluble fraction was lyophilized and was resuspended in methanol to prepare 100 mg/ml stock solution.

3.2.4 Sephadex G-10 column purification
The Sephadex G-10 column was used to separate the molecules based on their molecular size from the solvent extracted part of the crude extract. The sephadex G10 column (50 X 4 cm long with the bed volume of 600 ml and water as mobile phase) was prepared using one volume of Sephadex G-10 beads resuspended in two volumes of distilled water to prepare gel slurry. The gel slurry was loaded into the column having glass wool at the bottom. The column was stabilized with the flow of two to three bed volumes of double distilled water. Pestle of the column was opened to release excess of water present above the bead level. Sample was loaded into the column and around 200 ml volume was discarded (void volume) as this elute does not have any compounds. Then twenty fractions of 10 ml each were collected, lyophilized and stored at 4 °C. The lyophilized fractions were resuspended in RPMI medium to prepare 100 mg/ml stock solutions and were stored at 4 °C.

3.2.5 Thin layer chromatography (TLC)
Thin layer chromatography is used to separate the solutes based on their differential partition between the stationary and mobile phase. Aluminum sheet with silica gel layer (Merck) was used as a chromatographic plate and samples were spotted from the bottom of TLC plate using capillary tubes with a distance of 1.5 cm. The bottom of TLC plate was placed in a chamber pre-saturated with solvent (n-butanol/acetic acid/water in 4:1:1 ratio) to a depth of 1.5 cm and the solvent front was run till it reaches to the other end of TLC plate. TLC plate was sprayed with 0.1% ninhydrin in acetone and was dried at 100 °C for 10 min.

3.2.6 Reverse Phase High performance liquid chromatography
Reverse phase high performance liquid chromatography (RP-HPLC) was used to analyze the purity of bioactive fraction. The C-18 column was used in RP-HPLC and was de-gassed to eliminate the formation of bubbles and was calibrated with
0.1% Trifluoroacetic acid solvent to ensure the absence of detectable signals in UV-VIS absorption spectrum. Then a 2 ml of sample was injected into the sample loop of HPLC and sample was run into the column with the flow rate of 2 ml/min. After the elution of void volume, presence of analytes in the column was recorded by detecting a change in the absorption spectrum at a set wavelength of 225 nm.

### 3.2.7 Structural determination experiments

NMR and Mass spectroscopic analysis were performed to determine the structure of active molecule with the help of Dr. Anil Chatterji and Dr. P. S. Parameshwaran at National Institute of Oceanography (NIO), Goa, India and Dr. Raj Mohan from National Chemical Laboratory (NCL), Pune, India. In this, $^1$H and $^{13}$C NMR spectra were recorded on a Brucker AC-200 spectrophotometer and Mass spectroscopic analysis was performed using LC-MS (TOF) (Applied Biosystems, USA).

### 3.2.8 Synthesis of Zinc diprolinate

To prepare Zinc diprolinate, L-Proline (1.15 g in 10 ml of water) solution was drop-wise added to the Zinc acetate (3.6 g in 10 ml of water) or Zinc chloride.
(2.65 g in 10 ml water) solution. The mixture was stirred for 10 minutes at room temperature and then gradually heated up to 100 °C over a period of 6 hours. The reaction mixture was then allowed to cool at room temperature, lyophilized and was stored at 4 °C. The reaction was depicted in the following equation (Figure-3.1).

3.3 Results

3.3.1 Fractionation of Black Clam crude extract

Based on the results of antiviral screening assays described in section 2.3, Black Clam (Villorita cyprinoides) crude extract was selected for further purification. The bioactivity-guided purification was used to isolate the active component in the Black Clam crude extract. The Black Clam crude extract was separated into two fractions using solvent extraction method (Figure-3.2). The powdered crude extract of Black Clam as described in section 2.2.2 was extracted with methanol and the insoluble residue was separated to prepare methanol soluble part of Black Clam methanolic extract (BC-Me). The methanol insoluble part was vacuum dried and was suspended in water to prepare aqueous fraction of Black Clam aqueous extract (BC-Aq). BC-Me and BC-Aq were vacuum dried and were redissolved in methanol and water respectively to prepare 100 mg/ml stock solutions.

Fig 3.2: Flow chart of solvent extraction mediated fractionation of Black Clam crude extracts.

3.3.2 Determination of non-cytotoxic concentrations of BC-Aq and BC-Me fractions

The BC-Me and BC-Aq fractions of Black Clam crude extract were tested for their non-cytotoxic concentrations in CEM-GFP cells. Varied concentrations of
BC-Me and BC-Aq fractions ranging from 100 µg to 500 µg/ml were tested in CEM-GFP cells and percentage of cell viability was calculated by MTT cytotoxicity assay as described in section 2.2.3. The highest non-cytotoxic concentration of BC-Me at which more than 95% of cells were viable was determined as 300 µg/ml. The highest non-cytotoxic concentration of BC-Aq was determined to be 425 µg/ml (Figure-3.3).

3.3.3 Anti-HIV-1 activity of BC-Aq and BC-Me in HIV-1 infected T cells

The BC-Me and BC-Aq fractions of Black Clam crude extract were tested for their anti-HIV-1 activity in CEM-GFP cells. The cells were infected with HIV-1 NL4-3 at 0.1 MOI and were treated with 300 µg/ml of BC-Me and BC-Aq. The infected cells were harvested when more than 90% of cells were green in untreated cells under the fluorescence microscope. The p24 analysis data of supernatant indicated that virus production was decreased to more than 75% in BC-Me and 95% in BC-Aq treatments (Figure-3.4). The HIV-1 inhibitory effect of BC-Me was less than BC-Aq in three independent experiments as shown in Figure-3.4. This data indicated that BC-Aq inhibits HIV-1 replication in CEM-
Fig 3.4: Anti-HIV-1 activity of methanol (BC-Me, 300 μg/ml) and water soluble fraction (BC-Aq, 300 μg/ml) of Black Clam crude extract in CEM-GFP cells. UT, Un treated; AZT, Azidothymidine (5 μM).

GFP cells better than BC-Me. Based on these results, BC-Aq fraction was selected for further purification process.

3.3.4 Purification of BC-Aq fraction using Sephadex G-10 Column

The BC-Aq was passed through a Sephadex G-10 column to separate the active components based on their molecular size. The column was prepared as described.

Fig 3.5: Thin layer chromatographic profile of fractions of BC-Aq purified by Sephadex G-10 column (A to S). The samples were pooled based on their similar profile in TLC and are indicated as A to S.
in section 3.2.3. From the Sephadex G-10 column, 20 fractions of 10 ml each were collected after discarding the void volume. An aliquot of all fractions were run on thin layer chromatographic plate to check their profile. Based on TLC profile, 20 fractions were pooled into 8 fractions of BC Aq1 to 8 as shown in Figure-3.5, lyophilized and were resuspended in serum free RPMI medium to prepare 50 mg/ml stock solutions and were stored at 4 °C.

3.3.5 Anti-HIV-1 activity of BC-Aq1 fractions in T cell lines
The fractions BC-Aq1 to 8 were tested for their anti-HIV-1 activity in CEM-GFP cells infected with HIV-1NL4-3 using 50 μg/ml concentration. After the progress of HIV-1 infection, cells were assayed for GFP expression. The GFP analysis of infected CEM-GFP cells indicated that BC-Aq1 fraction inhibits HIV-1 replication. The detection of p24 antigen in culture supernatants using p24 ELISA showed that BC-Aq1 reduces virus production to more than 90% in HIV-1NL4-3 infected CEM-GFP cells. The BC-Aq1 was the most active fraction amongst BC-Aq1 to BC-Aq8 as shown in Figure-3.6. This result indicated that BC-Aq1 fraction contains the active component that efficiently inhibits HIV-1 replication as compared to other fractions.

![Graph](image)

Fig 3.6: Anti-HIV-1 activity of fractions of Black Clam Aqueous extract (BC-Aq1 to 8) in HIV-1NL4-3 infected CEM-GFP cells using p24 ELISA.
3.3.6 Detection of the purity of BC-Aq1 fraction

As BC-Aq1 was found to be the most active fraction, it was selected for further purification. However, as it showed a single band in TLC profile (Figure-3.5),

![HPLC Chromatogram of BC Aq1. The parameters of major peak: (i) Retention time: 32.06 min; Height: 1831.32 mA and Relative area: 82.52%.

Fig 3.7: HPLC Chromatogram of BC Aq1. The parameters of major peak: (i) Retention time: 32.06 min; Height: 1831.32 mA and Relative area: 82.52%.

The purity of BC-Aq1 was then determined by reverse phase high performance liquid chromatography (RP-HPLC). C18 column was used in RP-HPLC as described in 3.2.5. In HPLC chromatogram, a sharp single major peak with the retention time of 32.06 min, height of 1831.32 mA and 82.52% of relative area was observed (Figure-3.7). All the other minor peaks were less than 92.6 mA in height and with very low relative areas as shown in Figure-3.7. The sample was run for the second time to reconfirm the purity and similar profile was observed in HPLC.

3.3.7 Structural analysis of the active molecule in BC-Aq1

As BC-Aq1 was found to be a pure and active fraction, it was subjected to NMR and Mass spectroscopic (MS) analysis to determine the structure of the bioactive molecule. NMR and MS analysis were performed with the help of Dr. P. S.
Parameshwaran at NIO and Dr. Raj Mohan at NCL as described in 3.2.7. BC-Aq1 showed broad $^1$H ppm (Figure-3.8). The broad peaks in NMR spectrum indicated the presence of metal atom in the structure of the active molecule. The Mass spectrum of BC-Aq1 in an LC-MS/MS (High resolution) analysis had a major peak at m/z 293 (M + H$^+$) followed by minor peaks at 609 (2M + Na$^+$), 315 (1M + Na$^+$), 180 (M-Proline + H$^+$) and 116 (Proline + H$^+$). The isotopic peaks were in close proximity with m/z 293 such as 295 and 297 etc. The accurate mass of the molecule was calculated to be 293.028 (Figure-3.9). Its elemental composition was deduced as C$_{10}$H$_{16}$N$_2$O$_4$Zn using MS calculator, which corresponds to the structure, Diprolinato Zinc or Zinc diprolinate. Additional peaks after Zinc diprolinate (Mol. wt. 293) of 295 and 297 correspond to Zinc diprolinate having 66 and 68 Mol. wt. isotopes of Zinc metal atom in its structure. In general, Zinc metal atom has three isoforms with molecular weights of 64, 66 and 68. In this mass spectroscopic analysis of the molecule, such pattern was clearly observed, confirming the presence of Zinc metal atom in the identified structure. From the complementary structural analysis of BC-Aq1 and elemental composition analysis, the molecule was identified as C$_{10}$H$_{16}$N$_2$O$_4$Zn (Zinc diprolinate).
Fig 3.9: Mass spectrum of BC-Aq1. The mass of active molecule was calculated to be 293.05.

3.3.8 Anti-HIV-1 activity of BC Aq1 in T and monocytic cell lines

The pure and active BC-Aq1 was further tested in other T and monocytic cell lines. Cells were infected with HIV-1_{NL4-3} and cells were treated with 50 μg/ml concentration of BC-Aq1. After the progress of HIV-1 infection, the culture supernatants were assayed for virus production using p24 ELISA. BC-Aq1 inhibited more than 90% of virus release in HIV-1_{NL4-3} infected CEM-CM3 cells as compared to the untreated controls (Figure-3.10 A). Similar effect was observed in another T cell line Jurkat, in which, BC-Aq1 inhibited 75% of virus production (Figure-3.10 B). HIV-1 also infects monocytic cells and replicates in these cells to produce large number of infectious virions. After detection of HIV-1 inhibitory activity in T cells, BC-Aq1 was also tested in U937 and THP-1 monocytic cells. U937 cells were infected with 0.5 MOI of HIV-1_{NL4-3} and were treated with 50 μg/ml of BC-Aq1. The p24 ELISA results showed that BC-Aq1
Fig 3.10: Effect of BC-Aq1 on HIV-1\textsubscript{NL4-3} replication in (A) CEM-CM3 and (B) Jurkat T cells; (C) U937 and (D) THP-1 monocytic cells. UT, Untreated, BC-Aq1 (50 μg/ml) and AZT, Azidothymidine (5 μM).

inhibits more than 70% virus production in U937 cells (Figure-3.10 C). To further confirm our observations in monocytic cells, THP-1, another monocytic cell line, was infected with HIV-1\textsubscript{NL4-3} at 0.5 MOI and infected cells were treated with 50 μg/ml BC-Aq1. In the presence of BC-Aq1, more than 80% decrease in virus production was observed in HIV-1 infected THP-1 cells (Figure-3.10 D). These results confirm that BC-Aq1 inhibits HIV-1 replication in all the T and monocytic cells tested.

3.3.9 Synthesis of Zinc diprolinate and its structural analysis
As Zinc diprolinate is a small molecule and amenable to synthesis, we wanted to synthesize the molecule and then confirm the anti-HIV-1 activity. C\textsubscript{10}H\textsubscript{16}N\textsubscript{2}O\textsubscript{4}Zn (Zinc diprolinate) was synthesized with the help of Dr. P. S. Parameshwaran at National Institute of Oceanography, Goa, India as described in section 3.2.7. Zinc
diprolinate was synthesized by mixing Zinc acetate (dihydrate) with the amino acid L-Proline in 1:2 molar ratio by boiling the aqueous solution of the mixture. The structure of the synthesized Zinc diprolinate was confirmed using NMR and MS analysis. The $^1\text{H}$ NMR of the resulting product had broad peaks at $\delta 3.79$ (1H), 3.06 (2H), 2.1 (1H) and 1.7 (3H) accounting for all the C-H protons of L-Proline moiety (Figure-3.11). The broadness of peaks was due to the paramagnetic properties of Zinc ions. This was also supported by the carbon signals: $\delta 180$ (s), 62 (d), 48 (t), 30.8 (t) and 26 (t) as revealed by $^{13}\text{C}$ NMR experiments. Here also peaks were very broad due to the influence of paramagnetic metal ion (Zinc). The positive ionization mass spectrum of the compound using LC-MS (TOF) (Applied Biosystems, USA) displayed the base peak at m/z 293 ($\text{M} + \text{H}^+$) followed by minor peaks at m/z 766 ($3\text{M} – \text{Proline} + \text{H}$), 587 ($2\text{M} + \text{H}^+$), 472 ($2\text{M} – \text{Proline} + \text{H}^+$), 609 ($2\text{M} + \text{Na}^+$), 704 ($2\text{M} + \text{Proline}+\text{H}^+$), 315 ($1\text{M} + \text{Na}^+$), 180 ($\text{M} – \text{Proline} + \text{H}^+$) and 116 ($\text{Proline} + \text{H}^+$) (Figure-3.12). The negative ionization mass spectrum of Zinc diprolinate had base peak at m/z 114 corresponding to the amino acid Proline [- H]. In MS analysis, no
molecular ions were observed in the molecule because of internal charge neutralization. From this analysis, the synthesized compound was confirmed to be Zinc diprolinate.

3.3.10 Cytotoxicity of Zinc diprolinate in CEM-GFP cells

The cytotoxic profile of Zinc diprolinate was tested using different concentrations of Zinc diprolinate. In this, CEM-GFP cells were treated with 0.025, 0.05, 0.1, 0.2, 0.3 and 0.4 mM concentrations of Zinc diprolinate and percentage of viability was calculated using MTT cytotoxicity test as described in section 2.2.3. The
The synthetic molecule, Zinc diprolinate was tested in HIV-1\textsubscript{NL4-3} infected CEM-GFP cells. The 50% HIV-1 inhibitory concentration (IC\textsubscript{50}) of Zn(pr)\textsubscript{2} was also determined in HIV-1\textsubscript{NL4-3} infected CEM-GFP cells. In this, 2 X 10\textsuperscript{5} infected cells were treated with 10, 20, 40, 60, 80 and 100 μM concentrations of Zn(pr)\textsubscript{2} for 7 to 10 days. We observed dose dependent inhibition of HIV-1 production with increasing concentration of Zn(pr)\textsubscript{2}. Based on our results, the IC\textsubscript{50} value was calculated to be 47.67 μM (Figure-3.14). Zinc diprolinate inhibited more than 95% of HIV-1 production at 100 μM concentration (Figure-3.14). Zinc pipercolinate had no effect on HIV-1 replication at 100 μM concentration. This indicated that Zinc diprolinate is a specific inhibitor of HIV-1 replication. This also confirms that the anti-HIV-1 property of Zn(pr)\textsubscript{2} is specific to its unique structure. The anti-HIV-1 activity of Zn(pr)\textsubscript{2} was also tested in HIV-1\textsubscript{NL4-3}

![Graph showing cytotoxicity profile of Zinc diprolinate in CEM-GFP cells. The 50% cytotoxicity concentration (CC\textsubscript{50}) was calculated to be 0.242 mM.](image)
Fig 3.14: Zn(pr)$_2$ inhibits virus production in HIV-1$_{NL4,3}$ infected CEM- GFP cells in a dose dependent manner. The 50% inhibitory concentration (IC$_{50}$) of Zn(pr)$_2$ is 47.67 μM.

Fig 3.15: Effect of Zn(pr)$_2$ on HIV-1 replication in HIV-1$_{NL4,3}$ infected Jurkat T cells. UT, Untreated; Zn(Pr)$_2$, Zinc diprolinate (100 μM); NC, Negative control (Zinc pipecolinate, 100 μM); AZT, Azidothymidine (5 μM).

infected Jurkat cells to confirm the observed effect in another T cell line. Zn(pr)$_2$ treatment significantly reduced the virus release into the culture supernatant of HIV-1$_{NL4,3}$ infected Jurkat cells. L-proline at 100 μM did not show any activity. Also treatment with Zinc pipecolinate, a Zinc salt of Pipecolinic acid (Higher
homologue of L-Proline) had no effect on HIV-1 replication (Figure-3.15). These results show that Zn(pr)$_2$ specifically inhibits HIV-1 replication in T cells.

### 3.3.12 Anti-HIV-1 activity of Zinc diprolinate in HIV-1$_{IIB}$ infected T cells

Zinc diprolinate was then tested for its effect on HIV-1$_{IIB}$ replication in CEM-GFP cells. In this, CEM-GFP cells were infected with 0.1 MOI of HIV-1$_{IIB}$ and the virus produced into the culture supernatant was quantitated by p24 ELISA. More than 75% decrease in HIV-1$_{IIB}$ production was observed with 100 $\mu$M
Conc. of Zn(pr)$_2$, where as no inhibition was observed with Zinc pipecolinate (Figure-3.16). These results were further confirmed by testing the effect of Zn(pr)$_2$ on HIV-1$_{IIIb}$ infected Jurkat T cells. The quantitation of virus released into the supernatant showed that Zn(pr)$_2$ inhibits HIV-1$_{IIIb}$ replication in Jurkat cells (Figure-3.17). These results confirm that Zn(pr)$_2$ inhibits HIV-1 replication of different strains of subtype B viruses in T cells.

3.3.13 Anti-HIV-1 activity of Zinc diprolinate in HIV-1$_{NL4-3}$ infected monocytic cells

As Zn(pr)$_2$ inhibited HIV-1 replication in two different T cells, it was then evaluated for its effect on monocytic cell lines. We have infected U937 cells with 0.5 MOI of HIV-1$_{NL4-3}$ and cells were treated with Zn(pr)$_2$ at 100 $\mu$M concentration. More than 85% inhibition in virus production was observed with Zn(pr)$_2$ in HIV-1$_{NL4-3}$ infected U937 cells as compared to the untreated controls (Figure-3.18). Zinc pipecolinate had no effect on HIV-1 replication. From this data, it was clear that Zn(pr)$_2$ inhibits HIV-1 replication in both T and monocytic cells.

Fig 3.18: Anti-HIV-1 activity of Zinc diprolinate in HIV-1$_{NL4-3}$ infected U937 cells. UT, Untreated; Zn(Pr)$_2$, Zinc diprolinate (100 $\mu$M); NC, Negative control (Zinc pipecolinate, 100 $\mu$M); AZT, Azidothymidine (5 $\mu$M).
3.3.14 Zinc diprolinate inhibits pIndie-C1 (subtype C HIV-1 isolate) viral replication in CEM-GFP-CCR5 cells

The B and C subtypes of HIV-1 infect large number of people and contribute to majority of HIV-1 infections in the world. The viral tropism and mechanism of viral replication differ from one subtype to other subtypes. To observe the effect of Zn(pr)2 on subtype C HIV-1 infection, CEM-GFP-CCR5 cells were infected with HIV-1_{Indie-C1}, a subtype C HIV-1 isolate from India (Mochizuki et al, 1999) and were treated with 100 $\mu$M of Zn(pr)2. The treated cells showed very low level of GFP expression. The virus quantitation in culture supernatants showed more than 80% decrease in virus production in presence of Zn(pr)2 as compared to the untreated and Zinc pipercolinate treated control cells (Figure-3.19), which indicates that Zn(pr)2 also inhibits subtype C viral replication.

![Graph showing anti-HIV-1 activity of Zn(pr)2 in Indie-C1 (Subtype C virus) infected CEM-GFP-CCR5 cells. UT, Untreated; Zn(Pr)2, Zinc diprolinate (100 $\mu$M); NC, Negative control (Zinc pipercolinate, 100 $\mu$M); AZT, Azidothymidine (5 $\mu$M).]

3.3.15 Inhibitory effect of Zinc diprolinate on different MOI of HIV-1_{NL4-3} infection

As most of the antiviral assays were performed with 0.1 MOI of viruses, the effect of Zinc diprolinate on different multiplicity of infection (MOI) of HIV-1_{NL4-3} was tested in CEM-GFP cells. In this, CEM-GFP cells were infected with different MOI (0.01, 0.1, 1) of HIV-1_{NL4-3} and were treated with Zn(pr)2 and the production
Fig 3.20: Effect of Zinc diprolinate on different MOI of HIV-1\textsubscript{NL4-3} infected CEM-GFP cells. A, 0.01 MOI; B, 0.1 MOI and C, 1 MOI. UT, Untreated; Zn(Pr)$_2$, Zinc diprolinate (100 \( \mu \)M); NC, Negative control (Zinc pipecolinate, 100 \( \mu \)M); AZT, Azidothymidine (5 \( \mu \)M).

Fig 3.21: Effect of Zinc diprolinate on day 1, 3, 5, 7, 9 of 0.1 MOI infected CEM-GFP cells. Zn(Pr)$_2$, Zinc diprolinate (100 \( \mu \)M); NC, Negative control (100 \( \mu \)M of Zinc pipecolinate).
of virus was quantitated using p24 ELISA. In both 0.01 and 0.1 MOI virus infected cells, Zn(pr)$_2$ inhibited more than 90% virus production as compared to the untreated and Zinc pipecolinate treated control cells (Figure-3.20 A and B). In case of 1 MOI infected cells, around 60% inhibition of viral replication was observed with Zn(pr)$_2$ (Figure-3.20 C). We have also performed the analysis of p24 antigen in culture supernatants of day 1, 3, 5, 7 and 9 HIV-1$_{NL4-3}$ infected (0.1 MOI) CEM-GFP cells to observe the time dependent inhibition of Zn(pr)$_2$ on HIV-1$_{NL4-3}$ infection. From the beginning of day 1 to day 9, Zn(pr)$_2$ showed its inhibitory effect on HIV-1 replication (Figure-3.21).

3.3.16 Zinc diprolinate inhibits HIV-1 replication of ddC resistant and Protease inhibitor resistant viral mutants in T cells

The current antiretroviral drugs that are used to treat HIV-1 infected patients frequently fail to decrease the viral infection because of the generation of drug resistant variants. Novel anti-HIV-1 compounds with inhibitory effect on known drug resistant variants are thought to be useful for developing of novel therapeutics. The inhibitory effect of Zinc diprolinate was tested with HIV-

![Fig 3.22: Anti-HIV-1 activity of Zinc diprolinate in CEM-GFP cells infected with A) HIV-1$_{JF4A}$ (ddC drug resistant mutant) and B) HIV-1$_{L10R/M461/I63P/V82T}$ (Protease inhibitor drug resistant mutant). UT, Untreated; Zn(pr)$_2$, Zinc diprolinate (100 μM); NC, Negative control (100 μM of Zinc pipecolinate), AZT, Azidothymidine (5 μM).}
IL10R/M461/I63P/V82T, a protease inhibitor resistant viral mutant and HIV-1JF4A, a ddC drug resistant mutant in CEM-GFP cells to observe the effect of the compound on drug resistant mutant viral replication. Zinc diprolinate inhibited HIV-1 replication of both ddC resistant and Protease inhibitor resistant mutants. More than 80% decrease in virus production was observed in the culture supernatants of Zn(pr)₂ treated CEM-GFP cells infected with both HIV-1JF4A and HIV-1L10R/M461/I63P/V82T (Figure-3.22 A and B).

3.3.17 Zinc diprolinate inhibits HIV-1NL4-3 replication in human PBMCs

Zinc diprolinate efficiently inhibits HIV-1 replication in T and monocytic cell lines with different viral isolates. This indicates that Zn(pr)₂ is a potent inhibitor of HIV-1 replication. It was then tested in Phytohemagglutinin (PHA, 2 μg/ml) activated human PBMCs infected with HIV-1NL4-3 virus at 0.1 MOI. The infection was continued for 8 to 10 days in the presence and absence of 100 μM Zn(pr)₂. The treatment with Zn(pr)₂, decreased the HIV-1 production to more than 75% in hPBMCs. Where as Zinc pipercolinate had no effect on viral replication. The experimental results with hPBMCs with three healthy donors showed that Zn (pr)₂ inhibits HIV-1 replication in hPBMCs (Figure-3.23).

![Graph showing the anti-HIV-1 activity of Zn(pr)₂ in HIV-1NL4-3 infected human PBMCs.](image-url)

**Fig 3.23:** *Anti-HIV-1 activity of Zn(pr)₂ in HIV-1NL4-3 infected human PBMCs.*  
UT, Untreated; Zn(Pr)₂, Zinc diprolinate (100 μM); NC, Negative control (Zinc pipercolinate, 100 μM); AZT, Azidothymidine (5 μM).
3.3.18 Effect of Zinc diprolinate on subtype C HIV-1 primary isolate infected hPBMCs

The therapeutic potential of Zinc diprolinate was then tested against the infection of HIV-1 subtype C primary isolates obtained from two of the Indian patients (NARI, Pune, India). 5 ng of primary viral isolates VB 51 and VB 52 was used to infect 2.5 million hPBMCs. Zinc diprolinate inhibited more than 70% virus production in both of the primary isolate infected hPBMCs (Figure-3.24). These results indicated that Zinc diprolinate inhibits viral replication of both HIV-1 laboratory as well as primary isolates.

![Graph showing anti-HIV-1 activity of Zinc diprolinate in human PBMCs infected with A) VB51 and B) VB52 HIV-1 primary isolates. UT, Untreated; Zn(pr)₂, Zinc diprolinate (100 μM); NC, Negative control (Zinc pipecolinate, 100 μM), AZT, Azidothymidine (5 μM).]

3.3.19 Effect of Zinc diprolinate in long-term cultures of HIV-1NL4-3 infected CEM-GFP cells

The effectiveness of an antiretroviral drug is to show its effect for long time with out any failure to suppress HIV-1 replication. This is possible only when no drug resistant variants emerge during the treatment of HIV-1 infected people with the drug. The available drug treatment frequently lead to the emergence of drug resistant variants and fail to suppress the plasma viremia for long time during the
treatment. Thus 0.1 MOI of HIV-1$_{NL4-3}$ infected CEM-GFP cells were treated with 100 μM concentration Zinc diprolinate for 45 days to observe the suppression of viral replication and the possibility of generation of drug resistant mutant variants in long term cultures. During the 45 days period, post infection, Zinc diprolinate treated CEM-GFP cells did not show any increase in background levels of green fluorescence. We have also analysed the production of virus by these cells using p24 ELISA, in which viral replication was constantly suppressed in the culture supernatants of Zinc diprolinate treated HIV-1$_{NL4-3}$ infected cells on day 1, 5, 10, 20, 30, 40 and 45 (Figure-3.25).

3.4 Discussion

Most of the drugs used in current medical practice are derivatives of natural products. The natural products are purified and identified from their sources using sequential purification strategies such as chromatographic methods and bioactivity-guided fractionation etc. The bioactivity-guided fractionation is a commonly used process to identify bioactive natural products (Pezzuto et al, 1997). Several novel chemical structures having anti-cancer property, antiviral activity etc. were identified from marine organisms using this method. This
particular approach has the advantage over other methods of isolation in that detected bioactivity of the natural product is sequentially screened with purified fractions in each step of purification process to identify the bioactive agent. In our present study, the bioactive compound in Black Clam extract, which has the potency to inhibit HIV-1 replication, was purified by bioactivity-guided fractionation.

The crude extract of Black Clam (*Villorita cyprinoides*) was solubilized in methanol and water to separate into two major fractions using solvent extraction method. In this, a large number of molecules are separated based on their solubility. The aqueous extract (BC-Aq) was more active as compared to the methanol-extracted fraction (BC-Me). The active aqueous BC-Aq fraction was separated into 20 fractions based on their molecular size using Sephadex-G10 column chromatography and fractions were pooled into eight fractions (BC-Aq1 to BC-Aq8) based on their TLC profile. The BC-Aq1 contained the bioactive component and showed potent anti-HIV-1 activity over other fractions in CEM-GFP cells. The BC-Aq1 inhibited HIV-1 replication in both T and monocytic cells, which indicated that the active component is separated into this fraction during the purification process. The purity of BC-Aq1 was analysed by High performance liquid chromatography and it was confirmed to be a highly pure fraction.

With the concluding evidences from the analysis of sample purity and bioactivity, BC-Aq1 fraction was confirmed as a potent anti-HIV-1 fraction with high purity. The structure of the active component in BC-Aq1 was analyzed with NMR and Mass spectroscopy. The $^1$H and $^{13}$C NMR showed broad peaks because of the paramagnetic nature of the analyte, indicating the presence of metal atom in the structure of the molecule. Mass spectroscopic analysis of BC-Aq1 was performed to determine the molecular mass of active component. The active component has a molecular weight of 293.05 (M + H) and the other peaks were determined as the peaks of L-Proline (116.06), 180 (M – Proline + H$^+$), 315 (1M + Na$^+$), 609 (2M + Na$^+$), with low level of impurities. The elemental composition of the compound with 293.05 Mol. wt was deduced as C$_{10}$H$_{16}$N$_2$O$_4$Zn (Zinc diproline) with
minimal errors of – 0.2290 (mDa error) and – 0.7817 (ppm error) using elemental composition calculator as compared to the other possible structures like C₅H₁₇N₄O₆Zn [+ 3.7936 (mDa error) and + 12.9454 (ppm error)] and C₁₁H₉N₄O₆ [-4.4606 (mDa error) and –15.2215 (ppm error)]. Zinc diprolinate molecule was previously reported as a racemization agent of L-Lactide and is the only report on this molecule in the literature (Kricheldorf et al, 1998). However, neither the presence of Zinc diprolinate in the extract of an organism nor bioactivity was previously reported. Zinc diprolinate was then synthesized and NMR and Mass spectra of the molecule shows similar structural details as the highly pure BC-Aq1 fraction.

The synthetic molecule of Zinc diprolinate inhibited HIV-1 replication of different laboratory viral isolates like HIV-1NL4-3 and HIV-1IIIB in T and monocytic cells, indicating the potential HIV-1 inhibitory effect of Zn(pr)₂. The different subtypes of HIV-1 vary in their genomic sequences, viral tropism and mechanism of replication (Pereira et al, 2000). The subtype B and C viruses infect large number of people in diversified geographical areas in the world. After observing the effect of Zinc diprolinate on replication of HIV-1NL4-3 and HIV-1IIIB subtype B viral isolates, it was also tested in subtype C viral replication using HIV-1Indic-C1 virus. Zn(pr)₂ efficiently inhibited HIV-1Indic-C1 viral replication, which indicates its inhibitory effect on other viral isolates. Furthermore, we have repeated these experiments with hPBMCs isolated from the blood of three healthy donors to ensure the inhibitory effect of Zn(pr)₂ and similar results were obtained in all the experiments with hPBMCs of different sources. Thus Zinc diprolinate inhibited a number of laboratory-adapted viral strains in cell culture experiments as described. The primary subtype C viral isolates were isolated from two HIV-1 infected Indian patients and they use CCR5 co-receptor (CCR5-tropic) for their entry. In antiviral screening experiments, Zinc diprolinate inhibited HIV-1 replication of both the subtype C primary isolates. This evidently indicates that Zinc diprolinate is a potential anti-HIV-1 lead molecule. Zinc pipercolinate was used as a negative control as it has structural similarity to the lead compound (Zinc salt of Pipercolinic acid, a higher homologue of L-Proline) but had no effect
on virus infected cells. This also shows that the observed activity of Zinc diprolinate is specific to its unique structure.

During the treatment of HIV-1 infected patients with available antiretroviral drugs, drug resistant HIV-1 variants are generated, which replicate in the presence of the drug. The main reason for emergence of drug resistant variants is low fidelity of reverse transcriptase enzyme. This leads to the emergence of mutant variants of HIV-1, which have the capacity to resist the action of treated drug. Use of high concentrations of an anti-HIV-1 drug in long-term cultures may lead to the emergence of drug resistant mutant HIV-1 strains. Zinc diprolinate was used at 100 μM concentration to treat 0.1 MOI infected CEM-GFP cells and we have not observed any increase in GFP fluorescence and viral replication following the day of infection till 45 days post infection. We finally conclude that Zinc diprolinate is an inhibitor of HIV-1 replication with potential therapeutic importance.

3.4 References


